

## **AMENDMENTS TO THE SPECIFICATION**

**Please delete lines 1-9 on page 1 of the specification and replace it with the following amended paragraph:**

This application is a continuation of U.S. Application No. 09/711,485 filed November 13, 2000, now U.S. Patent No. 6,649,742, which is a continuation of U.S. Application No. 08/839,765 filed April 15, 1997, now U.S. Patent No. 6,146,631, which is a continuation of U.S. Application No. 08/425,336 filed April 18, 1995, now U.S. Patent No. 5,621,083, which is a continuation of U.S. Application No. 08/064,691 filed May 12, 1993, now abandoned, which is a continuation-in-part of ~~co-pending~~U.S. Patent Application Serial-No. 07/988,430 filed December 9, 1992, now U.S. Patent No. 5,416,202, which is a continuation-in-part of ~~pending~~ U.S. Patent Application Serial-No. 07/901,707 filed June 19, 1992, now U.S. Patent No. 5,376,546, which in turn is a continuation-in-part of U.S. Patent Application Serial-No. 07/787,567 filed November 4, 1991, now abandoned.

**Please delete lines 31-37 on page 14 and 1-12 on page 15 of the specification and replace it with the following amended paragraph:**

The RIP analogs of the invention are particularly suited for use as components of cytotoxic therapeutic agents. Cytotoxic agents according to the present invention may be used *in vivo* to selectively eliminate any cell type to which the RIP component is targeted by the specific binding capacity of the second component. To form cytotoxic agents, RIP analogs may be conjugated to monoclonal antibodies, including chimeric and CDR-grafted antibodies, and antibody domains/fragments (e.g., Fab, Fab', F(ab')<sub>2</sub>, single chain antibodies, and Fv or single variable domains). Analogs of RIPs conjugated to monoclonal antibodies genetically engineered to include free cysteine residues are also within the scope of the present invention. Examples of Fab' and F(ab')<sub>2</sub> fragments useful in the present invention are described in co-pending, co-owned U.S. Patent Application Serial No. 07/714,175, filed June 14, 1991, (abandoned) and in International Publication No. WO 89/00999 published on February 9, 1989, which are incorporated by reference herein.

**Pl ase del te lines 26-35 on pag 18 of the specification and r place it with the following amended paragraph:**

As noted above, RIPs may preferably be conjugated or fused to humanized or human-engineered antibodies, such as he3. Thus, the present invention also provides novel proteins comprising ~~an~~ a humanized antibody variable domain which is specifically reactive with an human CD5 cell differentiation marker. Specifically, the present invention provides proteins comprising the he3 light and heavy chain variable regions as shown in SEQ ID NOS: 95 125 or 96 126, respectively. DNA encoding certain he3 proteins is shown in SEQ ID NOS: ~~67 and 68~~ 72 and 71.

**Please delete lines 36-37 on page 18 and 1-2 on page 19 of the specification and replace it with the following amended paragraph:**

In a preferred embodiment of the present invention, the protein comprising ~~an~~ a humanized antibody variable region is an intact he3 immunoglobulin deposited as ATCC HB 11206.

**Please delete lines 3-5 on page 19 of the specification and replace it with the following amended paragraph:**

Also in a preferred embodiment of the invention, the protein comprising ~~an~~ a humanized antibody variable region is a Fab or F(ab')<sub>2</sub> or Fab fragment.

**Please delete lines 6-14 on page 19 of the specification and replace it with the following amended paragraph:**

Proteins according to the present invention may be made by methods taught herein and in co-pending, co-owned U.S. ~~P~~atent ~~A~~pplication ~~N~~o. 07/808,464 (abandoned) ~~by Studnicka et al.~~ incorporated by reference herein; and modified antibody variable domains made by such methods may be used in therapeutic administration to humans either alone or as part of an immunoconjugate as taught in co-owned, co-pending U.S. Patent Application No. 07/787,567 (abandoned). ~~by Better et al.~~

**Please delete lines 10-33 on page 22 of the specification and replace it with the following amended paragraphs:**

FIGs. 10A and 10B are alignments of the consensus amino acid sequences for the subgroups of light {hK1 (SEQ ID No. 149) (human kappa light chain subgroup 1), kH3 hK3 (SEQ. ID No. 150) (human kappa light chain subgroup 3), hK2 (SEQ ID No. 151) (human kappa light chain subgroup 2), hL1 (SEQ ID No. 152) (human lambda light chain subgroup 1), hL2 (SEQ ID No. 153) (human lambda light chain subgroup 2), ~~HL3~~ hL3 (SEQ ID No. 154) (human lambda light chain subgroup 3), hL6 (SEQ ID No. 155) (human lambda light chain subgroup 6), hK4 (SEQ ID No. 156) (human kappa light chain subgroup 4), hL4 (SEQ ID No. 157) (human lambda light chain subgroup 4) and hL5 (SEQ ID No. 158) (human lambda light chain subgroup 5} and heavy chains {hH3 (SEQ ID No. 159) (human heavy chain subgroup 3), hH1 (SEQ ID No. 160) (human heavy chain subgroup 1) and hH2 (SEQ ID No. 161) (human heavy chain subgroup 2)}, respectively, of human antibody variable domains;

FIG. 11 sets out the nucleotide sequences of the oligonucleotides utilized in the construction of the genes encoding modified V/J-regions of the light and heavy chains of the H65 mouse monoclonal antibody variable domain; H65K-1: SEQ ID No. 117; HUH-K1: SEQ ID No. 141; HUH-K2: SEQ ID No. 142; HUH-K3: SEQ ID No. 143; HUH-K4: SEQ ID No. 121; HUH-K5: SEQ ID No. 122; HUH-G1: SEQ ID NO. 144; HUH-G2: SEQ ID No. 145; HUH-G3: SEQ ID No. 137; HUH-G4: SEQ ID No. 138; HUH-G5: SEQ ID No. 139; HUH-G6: SEQ ID No. 140; H65G-2S: SEQ ID No. 146; H65-G2: SEQ ID No. 85; H65K-2S: SEQ ID No. 116; JK1-HindIII: SEQ ID No. 87; and

FIGs. 12A and 12B are alignments of human light chain consensus hK1 (SEQ ID No. 149) and heavy chain consensus hH1 (SEQ ID No. 160) with the light and heavy chain sequences, respectively, of the variable domain of human antibody EU (SEQ ID Nos. 162 and 166), human antibody TAC (SEQ ID Nos. 163 and 167), human antibody TAC modified according to the present invention (prop) (SEQ ID Nos. 164 and 168) and human antibody TAC modified according to a different method (Que) (SEQ ID Nos. 165 and 169).

**Please delete lines 1-17 on page 42 of the specification and replace it with the following amended paragraph:**

#### D. Purification Of Recombinant Gelonin

Recombinant gelonin was purified by the following procedure: *E. coli* fermentation broth was concentrated and buffer-exchanged to 10 mM sodium phosphate at pH 7.0 by using an S10Y10 cartridge over a DC10 unit (Amicon); the concentrated and buffer-exchanged material was applied to a CM52 column (100 g, 5X10 cm). The column was washed with 1 L of starting buffer and eluted with a 0 to 300 mM NaCl gradient in starting buffer (750 ml total volume). The pure gelonin containing fractions were pooled (elution was from 100-250 mM NaCl), concentrated over an Amicon YM10 membrane, equilibrated with 10 mM sodium phosphate buffer, pH 7.0, and stored frozen at -20°C. A further purification step was attempted using Blue ~~Toyopearl~~TOYOPEARL® chromatography. However, this procedure did not result in an increased purity of material and resulted in an approximate 50% loss of the starting material.

**Please delete lines 15-34 on page 43 of the specification and replace it with the following amended paragraph:**

Two analogs of gelonin were constructed in which one of the native gelonin cysteines that participates in an endogenous disulfide bond was replaced with a non-cysteine residue. Specifically, the cysteine at position 50 was replaced with an alanine residue, creating a gelonin analog (designated Gel<sub>A50(C44)</sub>, shown in SEQ ID NO: 99) which has a cysteine available for disulfide bonding at position 44. The Gel<sub>A50(C44)</sub> analog has been referred to previously as Gel<sub>C44</sub> (see, e.g., co-owned, co-pending U.S. Patent Application Serial No. 07/988,430 (now U.S. 5,416,202), incorporated by reference herein). Conversely, the cysteine at position 44 was replaced with an alanine residue, resulting in an analog (designated Gel<sub>A44(C50)</sub>, shown in SEQ ID NO: 100) which has a cysteine available for disulfide bonding at position 50. The Gel<sub>A44(C50)</sub> analog has been referred to previously as Gel<sub>C50</sub> (see, e.g., co-owned, co-pending U.S. Patent Application Serial No. 07/988,430, (now U.S. 5,416,202) incorporated by reference herein). The combined series of the foregoing twelve analogs thus spans the entire length of the mature gelonin protein.

**Please delete lines 4-14 on page 53 of the specification and replace it with the following amended paragraph:**

Antibodies for use in constructing immunotoxins according to the present invention may be humanized antibodies, such as he3 and fragments thereof which display increased content of human amino acids and a high affinity for human CD5 cell differentiation marker. he3 is a humanized form of a mouse H65 antibody (H65 is a preferred monoclonal antibody for use in preparing humanized antibodies according to the present invention and is produced by hybridoma cell line XMMLY-H65 (H65) deposited on December 8, 1986 with the American Type Culture Collection in Rockville, Maryland (A.T.C.C.) and given the Accession No. HB9286).

**Please delete lines 31-38 on page 53 and 1-6 on page 54 of the specification and replace it with the following amended paragraph:**

Construction of humanized antibody variable domains according to the present invention and for use as components of immunotoxins may be based on a method which includes the steps of: (1) identification of the amino acid residues of an antibody variable domain which may be modified without diminishing the native affinity of the domain for antigen while reducing its immunogenicity with respect to a heterologous species; and (2) the preparation of antibody variable domains having modifications at the identified residues which are useful for administration to heterologous species. The methods of the invention are based on a model of the antibody variable domain described herein and in U.S. co-owned U.S. Patent Application No. 07/808,464 (abandoned), which predicts the involvement of each amino acid in the structure of the domain.

**Please delete lines 7-18 on page 54 of the specification and replace it with the following amended paragraph:**

Unlike other methods for humanization of antibodies, which advocate replacement of the entire classical antibody framework regions with those from a human antibody, the methods described herein and in U.S. co-owned U.S. Patent Application No. 07/808,464 (abandoned), introduce human residues into the variable domain of an antibody only in positions which are not critical for antigen-binding activity and which are likely to be exposed to immunogenicity-stimulating factors. The present methods are

designed to retain sufficient natural internal structure of the variable domain so that the antigen-binding capacity of the modified domain is not diminished in comparison to the natural domain.

**Please delete line 19 on page 54 through line 2 on page 56 of the specification.**

**Please delete lines 5-34 on page 56 of the specification and replace it with the following amended paragraph:**

The humanized H65 heavy chain containing the moderate risk residues was assembled by the following strategy. The moderate-risk expression vector was assembled from intermediate vectors. The six oligonucleotide sequences (oligos), disclosed in Figure 42 11 and labelled HUH-G11, HUH-G12, HUH-G3, HUH-G4, HUH-G5, and HUH-G6 (the sequences of HUH-G11 and HUH-G12 are set out in SEQ ID Nos. 131 and 132 and HUH-G3, HUH-G4, HUH-G5, and HUH-G6 are set out in SEQ ID NOS: 137-140) were assembled by PCR. Oligonucleotides containing the synthetic humanized antibody gene were mixed in pairs (HUH-G11 + HUH-G12, HUH-G3 + HUH-G4, and HUH-G5 + HUH-G6) in a 100 µl reaction with 1 µg of each DNA and filled in as described above. A portion of each reaction product was mixed in pairs (HUH-G11, 12 + HUH-G3, 4; HUH-G3, 4 + HUH-G5, 6), 2.5 U Taq was added and samples were reincubated as described above. The V-J region was assembled by mixing equal amounts of the HUH-G11, 12, 3, 4 reaction product with the HUH-G3, 4, 5, 6 product, followed by PCR with 0.5 ug of primers H65G-2S and H65-G2 as described above. The reaction product was cut with *Sa*II and *Bst*EI and cloned into the expression vector, similar to that described for heavy chain in Robinson *et al.*, *Hum. Antibod. Hybridomas* 2:84 (1991), generating pING4617. That plasmid was sequenced with Sequenase (USB, Cleveland), revealing that two residues were altered (a G-A at position 288 and a A-T at position 312, numbered from the beginning of the leader

sequence). The correct variable region was restored by substitution of this region from pING4612, generating the expected V-region sequence in pING4619.

**Please delete lines 11-35 on page 61 and 1-2 on page 62 of the specification and replace it with the following amended paragraphs:**

~~Figure 11, provides d~~Data showing relative binding of he3 and CH65 to CD5 on molt-4M cells in a competition binding assay. ~~These results demonstrate~~ assay demonstrate that the moderate-risk changes made in he3 IgG result in an antibody with a higher affinity than the chimeric mouse-human form of this antibody (cH65) for its target, CD5.

#### Example 7

##### Preparation of Gelonin Immunoconjugates

Gelonin analogs of the invention were variously conjugated to murine (ATCC HB9286) and chimeric H65 (cH65) antibody, cH65 antibody domains (including cFab, cFab' and cF(ab')<sub>2</sub> fragments), and humanized antibodies and antibody domains, all of which are specifically reactive with the human T cell determinant CD5. H65 antibody was prepared and purified by methods described in U.S. Patent Application Serial No. 07/306,433 (abandoned), *supra* and International Publication No. WO 89/06968, *supra*. Chimeric H65 antibody was prepared according to methods similar to those described in Robinson *et al.*, *Human Antibodies and Hybridomas*, 2:84-93 (1991), incorporated by reference herein. Chimeric H65 Fab, Fab', and F(ab')<sub>2</sub> proteins were prepared as described in Better, *et al.*, *Proc. Nat. Acad. Sci. (USA)*, 90: 457-461 (1993), incorporated by reference herein. Finally, he3 humanized antibodies were prepared according to the procedures described in U.S. Patent Application Serial No. 07/808,464, (abandoned) incorporated by reference herein.

**Please delete lines 28-37 on page 64 of the specification and replace it with the following amended paragraph:**

Following conjugation, unreacted M2IT linkers on the antibody were quenched with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction solution was then loaded onto a gel filtration column [Sephadex G-150

(Pharmacia) in the case of Gel<sub>C248</sub>, Gel<sub>C247</sub>, Gel<sub>C244</sub> and Gel<sub>C239</sub> and an AcA-44 column (IBF Biotechnics, France) in the case of Gel<sub>A50(C44)</sub> and Gel<sub>C10</sub>]. The reactions were run over the gel filtration columns and eluted with 10 mM Tris, 0.15M NaCl pH 7. The first peak off each column was loaded onto Blue ~~Toyopearl~~® TOYOPEARL® resin (TosoHaas, Philadelphia, Pennsylvania) in 10 mM Tris, 30 mM NaCl, pH 7 and the product was eluted with 10 mM Tris, 0.5 M NaCl, pH 7.5.

**Please delete lines 19-33 on page 65 of the specification and replace it with the following amended paragraph:**

Analog Gel<sub>C247</sub> and Gel<sub>A50(C44)</sub> were also conjugated to various chimeric ~~cH65Fab, cH65Fab' and cH65F(ab')<sub>2</sub>~~ and "human engineered" ~~he1-Fab, he2-Fab, he3-Fab, he1-Fab' and he1-F(ab')<sub>2</sub>~~ antibody fragments. Chimeric H65 antibody fragments may be prepared according to the methods described in International Publication No. WO 89/00999, *supra*. The DNA sequences encoding the variable regions of H65 antibody fragments that were human engineered (referring to the replacement of selected murine-encoded amino acids to make the H65 antibody sequences less immunogenic to humans) according to the methods described above in Example 5, are set out in SEQ ID NO: 69 (variable region of the kappa chain of he1 and he2), SEQ ID NO: 70 (variable region of the gamma chain of he1), SEQ ID NO: 71 (variable region of the gamma chain of he2 and he3) and SEQ ID NO: 72 (variable region of the kappa chain of he3).

**Please delete lines 22-35 on page 66 of the specification and replace it with the following amended paragraph:**

The conjugation reaction between the free thiol on Gel<sub>C247</sub> and the derivitized he1 Fab-M2IT-TNB, conditions were as follows. A 5-fold excess of the gelonin analog was added to activated he1 Fab-M2IT-TNB (both proteins were in 0.1M Na phosphate, 0.2M NaCl, pH7.5) and the reaction mixture was incubated for 3.5 hours at room temperature and then overnight at 4°C. Following conjugation, untreated M2IT linkers were quenched with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The



quenched reaction solution was loaded onto a gel filtration column (G-75) equilibrated with 10 mM Tris, 150 mM NaCl, pH 7. The first peak off this column was diluted to 30 mM NaCl with 10 mM Tris, pH7 and loaded on Blue ~~Toyopearl®~~TOYOPEARL®. The product was eluted with 10 mM Tris, 0.5 M NaCl, pH 7.5.

**Please delete lines 23-36 on page 67 and 1-2 of page 68 of the specification and replace it with the following amended paragraphs**

For the reaction between the free thiol on Gel<sub>C247</sub> and the derivitized he1 Fab'-M2IT-TNB, conditions were as follows. A 5.7-fold molar excess of gelonin was added to activated he1 Fab'-M2IT-TNB and the final salt concentration was adjusted to 0.25 M. The reaction mix was incubated for 1.5 hours at room temperature and then over the weekend at 4°C. Following conjugation, unreacted M2IT linkers were quenched with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction solution was loaded onto a gel filtration column (AcA54) equilibrated with 10 mM Tris, 250 mM NaCl, pH 7.5. The first peak off this column was diluted to 20 mM NaCl with 10 mM Tris, pH 7 and loaded on Blue BLUE-~~Toyopearl®~~ TOYOPEARL® which was equilibrated in 10 mM Tris, 20 mM NaCl, pH 7. The column was then washed with 10 mM Tris, 30 mM NaCl, pH 7.5. The product was eluted with 10 mM Tris, 1 M NaCl, pH 7.5.

**Please delete lines 1-9 on page 69 of the specification and replace it with the following amended paragraph:**

Following conjugation the excess M2IT linkers were quenched by incubation with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction was loaded onto a gel filtration column (G-75) equilibrated in 10 mM Tris, 0.15 M NaCl, pH 7. The first peak off this column was diluted to 30 mM NaCl with 10 mM Tris, pH 7, and loaded onto a Blue ~~Toyopearl®~~ TOYOPEARL® (TosoHaas) column. The product was eluted with 10 mM Tris, 1 M NaCl, pH 7.5.

**Please delete lines 5-15 on page 70 of the specification and replace it with the following amended paragraph:**

Following conjugation, the excess M2IT linkers were quenched by incubation with 1:1 mole cysteamine to linker for 15 minutes at room temperature. The quenched reaction was loaded onto a ~~GammaBind-G~~ GAMMABIND® G (immobilized protein G affinity resin, obtained from Genex, Gaithersburg, Maryland) equilibrated in 10 mM Na phosphate, 0.15 M NaCl, pH 7. It was eluted with 0.5 M NaOAc, pH 3 and neutralized with Tris. It was dialyzed into 10 mM Tris, 0.15 M NaCl, pH 7 overnight, then diluted to 30 mM NaCl with 10 mM Tris, pH 7 and loaded onto a ~~blueToyopearl®~~ BLUE TOYOPEARL® (TosoHaas) column. The product was eluted with 10 mM Tris, 1 M NaCl, pH 7.5.

**Please delete lines 20-31 on page 83 of the specification and replace it with the following amended paragraph:**

Human peripheral blood cells were obtained from lymphapheresis samples (HemaCare Corporation, Sherman Oaks, CA) or venous blood samples (Stanford University Blood Bank, Palo Alto, CA) collected from healthy donors. Blood cells were enriched for PBLs using Ficoll-Hypaque density gradient centrifugation (~~Ficoll-Paque®~~ FICOLL-PAQUE®; Pharmacia, Piscataway, New Jersey) and subsequently washed 4 times with PBS. Residual erythrocytes were lysed with RBC lysing buffer (16 µM ammonium chloride, 1 mM potassium bicarbonate, 12.5 µM EDTA) during the second wash. Cell viability in the final suspension was >95% as assessed by trypan blue dye exclusion.

**Please delete lines 11-37 on page 84 and 1-5 on 85 of the specification and replace it with the following amended paragraph:**

Untreated SCID mice were bled for determination of mouse Ig levels. Human PBL-injected mice were bled at various intervals for quantitation of human Ig and sIL-2R. Blood collection was from the retro-orbital sinus into heparinized tubes. Blood samples were centrifuged at 300 x g for 10 min, and plasma was collected and stored at -70°C. Mouse and human Ig were quantified using standard sandwich ELISAs. Briefly, flat-bottom microtiter plates (~~Maxisorp~~ MAXISORP® Immuno-Plates, Nunc, Roskilde,

Denmark) were coated overnight at 4°C with goat anti-mouse IgG+IgA+IgM (Zymed Laboratories, Inc., South San Francisco, California) or goat anti-human Igs (Tago, Inc., Burlingame, California) in bicarbonate buffer, pH 9.6. Plates were blocked for 2 hours at room temperature with 1% BSA in Tris-buffered saline, pH 7.5 (TBS), and then incubated at 37°C for 1 hour with standards or samples serially-diluted in TBS/1% BSA/0.05% Tween 20. Standards used were a monoclonal mouse IgG2a (IND1 anti-melanoma; XOMA Corporation, Berkeley, California) and polyclonal human Ig (Sigma Chemical Co., St. Louis, Missouri). Subsequently, plates were washed with TBS/Tween 20 and incubated at 37°C for 1 hour with alkaline phosphatase-conjugated goat anti-mouse IgG+IgA+IgM or goat anti-human Igs (Caltag Laboratories, South San Francisco, California). Detection was by measurement of absorbance at 405 nm following incubation with 1 mg/ml p-nitro-phenylphosphate (Sigma) in 10% diethanolamine buffer, pH 9.8. Plasma from a normal BALB/c mouse was used as a positive control in the mouse Ig ELISA. Plasma samples from naive SCID mice or normal BALB/c mice did not have detectable levels of human Ig. Human sIL-2R was quantified using an ELISA kit (Immunotech S.A., Marseille, France) as per the manufacturer's instructions.

**Please delete lines 26-36 on page 100 and line 1-7 on page 101 of the specification and replace it with the following amended paragraph:**

Plasmids pING4628 and pING4633 were transformed into *E. coli* E104. Bacterial cultures were induced with arabinose and cell-free supernatant comprising the he3Fab was concentrated and filtered into 20 mM HEPES, pH 6.8. The sample was then loaded onto a CM Sphradex column (2.5 x 3 cm), equilibrated in 20 mM HEPES, 1.5 mM NaCl, pH 6.8. The column was washed with the same buffer and eluted with 20 mM HEPES, 27 mM NaCl, pH 6.8. The eluate was split into 2 aliquots and each was loaded onto and eluted from a protein G (Bioprocessing) column (2.5 x 2.5 cm) separately. The protein G column was equilibrated in 20 mM HEPES, 75 mM NaCl, pH 6.8 and the sample was eluted with 100 mM glycine, 100 mM NaCl, pH 3.0. The two eluates were combined and diluted two times with 20 mM HEPES, 3 M ammonium sulfate, pH 6.8. The diluted eluates were loaded onto phenyl sepharose SEPHAROSE® high substitution Fast Flow (Pharmacia) column (2.5 x 3.3 cm), equilibrated in 20 mM

HEPES, 1.5 M ammonium sulfate, pH 6.8. The column was then eluted with 20 mM HEPES, 0.6 M ammonium sulfate, pH 6.8.

**Please delete lines 19-29 on page 109 of the specification and replace it with the following amended paragraph:**

Both pING3770 and pING3772 were transformed into *E. coli* (E104) cells by techniques known to those of ordinary skill in the art and induced with arabinose. Concentrated supernatants from the transformed cell cultures were analyzed by Western blot analysis with rabbit anti-gelonin antiserum. Transformants ~~from~~ from both plasmids generated a reactive band on the gel at the size expected for a Fab molecule carrying two gelonins (approximately 105 kD). These results are consistent with the production of fusion proteins comprising monovalent Fab, with both Fd and kappa chains separately fused to gelonin.

**Please delete lines 9-28 on page 113 of the specification and replace it with the following amended paragraph:**

Cell-free supernatant was passed through a CM ~~spheradex~~ SPHERADDEX® column (5 x 3 cm), equilibrated with 10 mM ~~Naphosphate~~ Na phosphate, pH 7.0. Single-chain antibody binds to the column which is then washed with 10 mM ~~Naphosphate~~ Na phosphate, 45 mM NaCl, pH 7.0. The fusion protein was then eluted with 10 mM ~~Naphosphate~~ Na phosphate, 200 mM NaCl, pH 7.0. The eluate was diluted two-fold with 20 mM ~~HePES~~ HEPES®, 3 M ammonium sulfate, pH 7.0 and loaded onto a butyl ~~sepharese~~ SEPHAROSE® Fast Flow (Pharmacia) column (2.5 x 4.1 cm) equilibrated in 20 mM ~~HEPES®~~, 1.5 M ammonium sulfate, pH 7.0. The column was then washed with 20 mM ~~HEPES®~~, 1.0 M ammonium sulfate, pH 7.0 and eluted with 20 mM ~~HEPES®~~ pH 7.0. The butyl ~~sepharese~~ SEPHAROSE® eluate was concentrated to a volume of 2-4 ml in an Amicon stirred cell fitted with a YM10 membrane. The concentrated sample was loaded onto an S-200 (Pharmacia) column (3.2 x 38 cm) equilibrated in 10 mM Na phosphate, 150 mM NaCl, pH 7.0. The column was then run in the same buffer and the fractions were collected. Some of the fractions were analyzed by SDS-PAGE to determine which fractions to pool together for the final product.

**Please delete lines 27-37 on page 117 of the specification and replace it with the following amended paragraph:**

Fusion proteins and immunoconjugates according to the present invention were used in a comparative cytotoxicity assay. Two assays were conducted, one targeting T cell line HSB2, and the other targeting lectin-activated peripheral blood mononuclear cells (PBMC) according to procedures in Example 6. The results of the assays are presented below in Tables ~~44a and 44b~~ 16a and 16b.

**Please delete lines 33-36 on page 122 and 1-15 on page 123 of the specification and replace it with the following amended paragraph:**

Native BRIP was purified from pearled barley flour. Four kilograms of flour ~~was~~were extracted with 16 liters of extraction buffer (10 mM ~~NaPO4~~ NaPO<sub>4</sub>, 25 mM NaCl, pH 7.2) for 20 hours at 4°C. The sediment was removed by centrifugation, and 200 ml of packed S-Sepharese~~SEPHARESE~~SEPHAROSE® (Pharmacia, Piscataway, New Jersey) was added to absorb BRIP. After mixing for 20 hours at 4°C, the resin was allowed to settle out, rinsed several times with extraction buffer and then packed into a 2.6 x 40 cm column. Once packed, the column was washed with extraction buffer (150 ml/h) until the absorbance of the effluent approached~~s~~ zero. BRIP was then eluted with a linear gradient of 0.025 to 0.3 M NaCl in extraction buffer and 5 ml fractions were collected. BRIP-containing peaks (identified by Western analysis of column fractions) were pooled, concentrated to about 20 ml, and then chromatographed on a 2.6 x 100 cm Sephacryl~~SEPHACRYL~~SEPHACRYL® S-200HR (Pharmacia) column equilibrated in 10 mM NaPO<sub>4</sub>, 125 mM NaCl, pH 7.~~[[4]]~~5 (10 ml/hr). BRIP-containing peaks were pooled again, concentrated, and stored at -70°C.

**Please delete lines 25-36 on page 123 and 1-4 on page 124 of the specification and replace it with the following amended paragraph:**

A cDNA expression library prepared from germinating barley seeds in the phage  $\lambda$  expression vector  $\lambda$ ZAPII was purchased from Stratagene, La Jolla, CA. Approximately 700,000 phage plaques were screened with anti-BRIP polyclonal

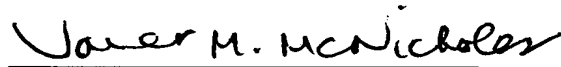
antisera and 6 immunoreactive plaques were identified. One plaque was chosen, and the cDNA contained therein was excised from  $\lambda$ ZAPII with *EcoRI* and subcloned into pUC18 generating the vector pBS1. The cDNA insert was sequenced with Sequenase SEQUENASE® (United States Biochemical, Cleveland, OhioOH). The DNA sequence of the native BRIP gene is set out in SEQ ID NO: 12. To confirm that cDNA encoded the native BRIP gene, the cDNA was expressed in the *E. coli* plasmid pKK233-2 (Pharmacia). BRIP protein was detected in IPTG-induced cells transformed with the plasmid by Western analysis with above-described rabbit anti-BRIP antisera.

This Preliminary Amendment is submitted in connection with the above-identified continuation application filed herewith. Please amend the continuation application in accordance with this Preliminary Amendment prior to any other actions on the merits.

Please charge any fees or credit any overpayment to the Deposit Account of McAndrews, Held & Malloy, Ltd., Account No. 13-0017.

Respectfully submitted,

DATED: November 18, 2003

  
Janet M. McNicholas, Ph.D.  
Reg. No. 32,918

McAndrews, Held & Malloy, Ltd.  
500 W. Madison St.,  
34<sup>th</sup> Floor  
Chicago, IL 60661  
(312) 775-8000